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Whole-profile soil organic matter content, composition, and stability under cropping systems that differ in belowground inputs

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Abstract

Subsoils have been identified as a potential carbon sink because they typically have low soil organic carbon (SOC) concentrations and high SOC stability. One proposed strategy to increase SOC stocks is to enhance C inputs to the subsoil by increasing crop rotation diversity with deep-rooted perennial crops. Using three long-term field trials in Iowa (study durations of 60, 35, and 12 years), we examined the effects of contrasting cropping systems [maize (*Zea mays* L.)-soybean (*Glycine max* (L.) Merr) (= two-year system) vs. maize-soybean-oat (*Avena sativa* L.)/alfalfa (*Medicago sativa* L.)-alfalfa or maize-maize-oat/alfalfa-alfalfa (= four-year system)] on above- and below-ground C inputs, as well as the content, biochemical composition, and distribution of SOC among physical fractions differing in stability to 90 cm depth. Average annual total C inputs were similar in the two-year and four-year systems, but the proportion of C delivered belowground was 20–35 % greater in the four-year system. Despite the long duration of these studies, the effect of cropping system on SOC content to 90 cm was inconsistent across trials, ranging from −7 % to +16 % in the four-year relative to the two-year system. At the one site where SOC was significantly greater in the four-year system, the effect of cropping system on SOC content was observed in surface and subsoil layers rather than limited to the subsoil (i.e., below 30 cm). Cropping system had minimal effects on biochemical indicators of plant-derived organic matter or on the proportions of SOC in labile particulate organic matter versus stable mineral-associated organic matter. We conclude that adoption of cropping systems with enhanced belowground C inputs may increase total profile SOC, but the effect is minimal and inconsistent; furthermore, it has minor impact on the vertical distribution, biochemical composition, and stability of SOC in Mollisols of the Midwest U.S.

Keywords

Subsoil, Carbon, Roots, Corn Belt

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ABSTRACT

Subsoils have been identified as a potential carbon sink because they typically have low soil organic carbon (SOC) concentrations and high SOC stability. One proposed strategy to increase SOC stocks is to enhance C inputs to the subsoil by increasing crop rotation diversity with deep-rooted perennial crops. Using three long-term field trials in Iowa (study durations of 60, 35, and 12 years), we examined the effects of contrasting cropping systems [maize (*Zea mays* L.)-soybean (*Glycine max* (L.) Merr) (= two-year system) vs. maize-soybean-oat (*Avena sativa* L.)/alfalfa (*Medicago sativa* L.)-alfalfa or maize-maize-oat/alfalfa-alfalfa (= four-year system)] on above- and below-ground C inputs, as well as the content, biochemical composition, and distribution of SOC among physical fractions differing in stability to 90 cm depth. Average annual total C inputs were similar in the two-year and four-year systems, but the proportion of C delivered belowground was 20–35 % greater in the four-year system. Despite the long duration of these studies, the effect of cropping system on SOC content to 90 cm was inconsistent across trials, ranging from −7 % to +16 % in the four-year relative to the two-year system. At the one site where SOC was significantly greater in the four-year system, the effect of cropping system on SOC content was observed in surface and subsoil layers rather than limited to the subsoil (i.e., below 30 cm). Cropping system had minimal effects on biochemical indicators of plant-derived organic matter or on the proportions of SOC in labile particulate organic matter versus stable mineral-associated organic matter. We conclude that adoption of cropping systems with enhanced belowground C inputs may increase total profile SOC, but the effect is minimal and inconsistent; furthermore, it has minor impact on the vertical distribution, biochemical composition, and stability of SOC in Mollisols of the Midwest U.S.

1. Introduction

Soil organic carbon (SOC) is an important pool in the global carbon (C) cycle, containing more C than the atmosphere and terrestrial biosphere combined (Schlesinger and Bernhardt, 2013). Soil organic C is also a key driver of cropland productivity through its influence on soil physical properties and plant nutrient supply (Weil and Magdoff, 2004). Through cultivation, drainage, crop removal, and other disturbances, agricultural land use has led to the loss of ~133 Pg of SOC globally (Sanderman et al., 2017). However, improved farming practices can be used to restore a portion of the SOC that was lost from agricultural soils, helping to sequester atmospheric CO_2 and improve soil quality (Paustian et al., 2016).

Soil organic C concentrations usually decline with depth, yet the amount of SOC found below 20 cm depth makes up approximately half of the SOC found within the top meter globally (Balesdent et al., 2018). Subsoils may store C more efficiently than topsoils because they typically have low C concentrations and high C stabilization capacity (Stewart et al., 2008). Subsoil C generally has an older radiocarbon age than topsoil C, indicating that a portion of organic C found in subsoils is stable over long timescales (Rumpel and Kögel-Knabner, 2011). Because of these features, subsoils have the potential to serve as large C sinks (Rumpel et al., 2012).

Subsoil C storage may be achieved by increasing C inputs to deep soil layers, decreasing the rate of SOC mineralization, or a combination of both. Carbon inputs are delivered to deep layers through root

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growth, bioturbation or mechanical incorporation of surface litters, and transport of dissolved organic C from surface layers (Rumpel and Kögel-Knabner, 2011). Management practices designed to increase subsoil C stocks have focused primarily on increasing root C inputs at depth (Paustian et al., 2016) for at least three reasons. First, the similarity of root and SOC vertical distributions across biomes suggests that root C inputs are a dominant control on deep SOC stocks (Fisher et al., 1994; Jobbagy and Jackson, 2000). Second, root-derived C has a longer residence time than shoot-derived C potentially because it is more biochemically resistant to mineralization (Ahmad et al., 2014; Rasse et al., 2005) and more likely to become protected through occlusion within soil aggregates than is shoot C (Denef and Six, 2006; Gale et al., 2000; Kong and Six, 2010; Six et al., 2002). And third, variation exists in root system size and depth within and among crop species, which can potentially be exploited for deep C delivery (Kell, 2012; Lynch and Wojciechowski, 2015).

The inclusion of deep-rooted perennial crops in crop rotations is one option to increase root C delivery to subsoils (Börjesson et al., 2018). Previous research showed greater belowground C inputs and whole-profile SOC stocks in cropping systems that included the perennial legume alfalfa (*Medicago sativa* L.) compared to annual grain systems, such as a maize (*Zea mays* L.)-soybean (*Glycine max* (L.) Merr) rotation (Gregorich et al., 2001; Russell et al., 2009, 2005; Sanford et al., 2012). While these studies suggest that deep-rooting cropping systems can increase SOC stocks, other research has shown no such effect (Bell et al., 2012). A better understanding of the impact of agricultural management on root inputs, and the effects of enhanced root inputs on subsoil C storage, will be required if deep-rooting cropping systems are to be widely adopted for C storage (Paustian et al., 2016).

The biochemical composition of soil organic matter and its distribution among physical fractions can reveal information about the sources and stability of SOC in different management systems. For example, the C/N ratio of soil organic matter indicates the extent of microbial processing because the C/N ratios of fresh plant inputs and microbial products are distinct (> 20 vs. 5–8 mass ratios, respectively) (Cleveland and Liptzin, 2007). Similarly, the ratio of mannose plus galactose to arabinose plus xylose indicates the relative abundance of microbially-derived to plant-derived sugars (Oades, 1984). In addition, the concentration of lignin, which is a relatively resistant polymer produced by plants (Kögel-Knabner, 2002), provides information about both the abundance of plant-derived compounds in the soil and the role of biochemical recalcitrance in organic matter preservation. The acid/aldehyde ratios of two lignin monomer classes - vanillyl and syringyl - reflect the oxidation state of lignin-derived phenols, with higher values indicating a greater extent of decomposition (Hedges et al., 1988). In addition to these biochemical indicators, physically-isolated fractions can be used to determine the contributions of labile, predominantly plant-derived organic matter (i.e., particulate organic matter; POM) and stable, more microbially-processed organic matter (i.e., mineral-associated organic matter; MAOM) found within or outside of aggregates (Lavallee et al., 2019; Six et al., 2002; von Lützow et al., 2006).

The purpose of the present study was to determine the effect of a four-year, alfalfa-inclusive cropping system on vertical patterns of SOC relative to a two-year maize-soybean system. We hypothesized that: 1) the four-year system would allocate a greater proportion of C inputs belowground resulting in greater SOC stocks, and 2) greater belowground inputs in the four-year system would result in an enrichment of fresh plant-derived organic matter and aggregate-occluded SOC, particularly in subsoils.

2. Materials and methods

2.1. Site descriptions

We conducted soil sampling in three long-term cropping systems experiments: at Kanawha, which was established in 1954 in northern Iowa (42° 94′N, 93° 17′W); at Nashua, which was established in 1979 in northeast Iowa (42° 95′N, 92° 54′W); and at Marsden, which was established in 2002 in central Iowa (42° 01′N, 93° 47′W), USA. All three experiments are on Iowa State University Research and Demonstration Farms. Mean annual precipitation is 818, 884, and 864 mm (1985–2014) and mean annual temperature is 8.03, 8.37, and 9.28° C for Kanawha, Nashua, and Marsden, respectively (Iowa State University, 2017). Soils at the Kanawha site are classified as Typic Endoaquolls (Webster series), soils at the Nashua site are predominantly Typic Hapludolls (Kenyon series), with a smaller area of Aquic Hapludolls (Readlyn series), and soils at the Marsden site are Typic Hapludolls (Clarion series), Aquic Hapludolls (Nicollet series), and Typic Endoaqolls (Webster series) according to the USDA Soil Taxonomy (Soil Survey Staff, 2018). At all sites, the cropping systems are rain-fed and have subsurface tile drainage systems (patterned tile at Kanawha and Nashua; irregular tiles at Marsden).

2.2. Experimental design

The experimental design at the Kanawha and Nashua sites is a randomized complete block split-plot design with rotation as the main plot factor, N fertilizer rate to maize as the split-plot factor, and two (Kanawha) or three (Nashua) replicate blocks. The experimental design at the Marsden site is a randomized complete block split-plot design with rotation as the main plot factor, weed management as the split-plot factor, and four replicate blocks. All three locations include a two-year maize-soybean system and a four-year maize-maize-oat/alfalfa-alfalfa or maize-soybean-oat/alfalfa-alfalfa system. All phases of each rotation are represented in each year.

The cropping system management practices for the three locations are summarized in Table 1. The two-year and four-year systems at Kanawha and Nashua differ only in the crop rotation, with nutrient inputs and tillage methods held constant between systems. In contrast, the four-year system at Marsden was designed to represent a diversified farming system typical of the Corn Belt, and thus differs from the two-year system in nutrient source and tillage method (Liebman et al.,

Table 1
Cropping system management at three long-term experiments in Iowa, USA.

	Two-year			Four-year		
Location (est'd)	Crop sequence[a]	Maize N rate (kg ha^{-1})	Primary tillage after maize[b]	Crop sequence[a]	Maize N rate (kg ha^{-1})	Primary tillage after maize/after alfalfa[b]
Kanawha (1954)	M-S	180	M	M-M-O/A-A	180	M/M
Nashua (1979)	M-S	180	C	M-M-O/A-A	180	C/C
Marsden (2002)	M-S	150	C	M-S-O/A-A	100–200[c]	C/M

[a] M = maize, S = soybean, O = oats, A = alfalfa. Oats were undersown with alfalfa. Maize, soybean, and oats are harvested for grain at all three locations and oat straw is also harvested at Marsden. Alfalfa hay is harvested in one cutting during the establishment year (Marsden only) and three or four cuttings the year after establishment (all locations).
[b] C = chisel plow, M = moldboard plow. Secondary spring tillage occurred before every crop except alfalfa.
[c] Applied as combination of composted cattle manure (~120 kg total N ha^{-1}) and fertilizer N.

Table 2

Selected soil properties by cropping system and depth at three long-term experiments in Iowa, USA. Standard errors are shown in parentheses. Different lowercase letters indicate significantly different values within a column for a particular location ($P < 0.10$). There were no significant differences in values between crop rotations for a given depth ($P < 0.10$).

Depth (cm)	Sand content (g 100 g^{-1} soil)		Clay content (g 100 g^{-1} soil)		Bulk density (g cm^{-3})	
	Two-year	Four-year	Two-year	Four-year	Two-year	Four-year
			Kanawha			
0–15	27.5 (1.0) a	26.7 (6.8) a	33.7 (0.4) a	36.8 (0.9) a	1.05 (0.04) d	0.99 (0.01) d
15-30	25.8 (2.7) a	25.5 (7.4) a	34.6 (1.5) a	35.4 (3.0) a	1.13 (0.02) c	1.10 (0.04) c
30-60	28.4 (5.6) a	23.6 (10) a	31.0 (1.7) a	34.7 (2.3) a	1.20 (0.01) b	1.17 (0.01) b
60-90	39.5 (11) a	25.8 (17) a	26.8 (5.3) a	36.5 (9.5) a	1.38 (0.05) a	1.27 (0.02) a
			Nashua			
0–15	31.2 (2.4) b	30.0 (1.6) b	27.0 (3.9) ab	28.7 (1.8) ab	1.22 (0.05) c	1.21 (0.06) c
15-30	29.1 (0.5) b	28.3 (1.3) b	25.4 (0.7) b	26.5 (2.5) b	1.38 (0.04) b	1.29 (0.02) b
30-60	42.7 (1.2) a	40.2 (2.5) a	27.2 (0.1) ab	31.3 (1.1) ab	1.37 (0.03) ab	1.37 (0.02) ab
60-90	44.5 (0.5) a	45.6 (2.2) a	29.8 (1.5) a	31.9 (1.3) a	1.52 (0.05) a	1.50 (0.14) a
			Marsden			
0–15	32.8 (3.3) b	35.4 (4.7) b	35.3 (3.0) a	34.1 (5.5) a	1.16 (0.02) c	1.13 (0.02) c
15-30	30.1 (4.6) b	35.5 (4.7) b	36.2 (4.0) a	32.9 (2.5) a	1.25 (0.04) bc	1.18 (0.02) c
30-60	33.8 (3.9) b	37.5 (5.8) b	36.7 (5.7) a	34.0 (3.2) a	1.36 (0.02) b	1.37 (0.03) b
60-90	38.4 (5.0) a	45.4 (6.0) a	31.3 (5.1) b	27.5 (3.5) b	1.55 (0.06) a	1.64 (0.06) a

2008). In particular, the four-year system derives a portion of nutrients from composted cattle manure and receives inversion tillage following alfalfa in addition to vertical tillage following maize. The four-year system at Marsden also differs from the four-year system at the other two locations because it includes soybeans as the second phase rather than maize, and oats are harvested for grain and straw rather than grain only.

At the Kanawha and Nashua sites, main plots were subdivided to accommodate four N fertilizer rates applied to maize, but we sampled only the 180 kg N ha^{-1} treatment. This N rate treatment has been in place since establishment of the experiment at Nashua and since 1984 at Kanawha (between 1954 and 1984, this treatment received 136 kg N ha^{-1} for maize). At the Marsden site, all phases of each rotation within each block were subdivided to accommodate two weed management treatments (conventional and low-herbicide) applied to maize and soybean, but we sampled only the conventional herbicide regime. The weed management treatments were in place since 2008; between 2002 and 2008, the two-year system received only conventional weed management and the four-year system received only low-herbicide weed management.

The experimental units measure 6.1 × 12.1 m at Kanawha, 4.6 × 15.2 m at Nashua, and 9 × 85 m at Marsden. Additional information about cropping systems, soil management, N fertilization, crop yields and site characteristics have been published previously (Hunt et al., 2017; Liebman et al., 2008; Mallarino et al., 2016a, 2016b; Robinson et al., 1996; Russell et al., 2005).

2.3. Carbon inputs

Average annual C inputs to soil in the two- and four-year systems were estimated for each year from 2003 to 2014 using yield data averaged across replicates for each crop within each rotation at the three locations (Supplementary Table 1). We used the allometric equations described by Bolinder et al. (2007) to transform yield data into above- and below-ground C inputs, and root distribution patterns from Fan et al. (2016) to estimate belowground C inputs above and below 30 cm depth. The C allocation coefficients and equations used to calculate C inputs are presented in Supplementary Tables 2 and 3. Rates of manure application and manure C concentrations from Marsden plot management records for 2003–2014 were used to account for manure C inputs to the four-year system at this site. The estimated C inputs by crop and depth of delivery are shown in Supplementary Table 4.

Although there is substantial uncertainty in estimates of belowground C inputs (Bolinder et al., 2007), we were able to verify a subset of our estimates using actual measurements taken at Kanawha and Nashua. We found that maize root dry matter inputs (not including rhizodeposits) for the two-year system calculated to 85 cm depth using the Bolinder and Fan equations were similar to measured root biomass to 85 cm (3.0 Mg root dry matter ha^{-1} estimated vs. 2.6 measured at Kanawha; 3.2 estimated vs. 2.4 measured at Nashua) (Russell et al., 2009). In addition, Keel et al. (2017) demonstrated that the Bolinder equations produced C input estimates that resulted in accurate simulation of SOC changes. Nevertheless, we performed a sensitivity analysis to determine changes in average annual C inputs and the proportion of C delivered belowground using variable assumptions for shoot: root ratios (Supplementary Table 5).

2.4. Soil sampling and characterization

Six 4-cm diameter cores were taken in each plot to a depth of 90 cm using a hydraulic soil probe following maize harvest and before autumn tillage in 2014. For the cropping systems that include two maize phases (four-year systems at Kanawha and Nashua), we sampled during the first maize phase. The cores were split into 0–15, 15–30, 30–60, and 60 − 90 cm depth increments, and the segments from the six cores within each plot were composited to form four samples per plot. The samples were weighed and a subsample was dried at 105^{0}C for moisture content determination. The sample dry weight and volume of cores were used to calculate bulk density. The remainder of each sample was passed through an 8-mm sieve and allowed to dry at room temperature. A portion of each air-dried sample was finely ground for determination of sand and clay content according to Kettler et al. (2001) (no gravels were present in the samples). Carbon and N concentrations were determined by dry combustion analysis (Vario Max CN analyzer, Elementar Americas, Mt. Laurel, NJ). Carbonates were removed prior to dry combustion analysis by the acid fumigation method (Harris et al., 2001). The C/N ratio of each sample was calculated as the organic C concentration to total N concentration (concentrations in units of g C or N 100 g soil^{-1}). Soil sand content, clay content, and bulk density are shown by depth for each cropping system and each site in Table 2.

2.5. Estimation of soil organic C stocks by the equivalent soil mass method

Soil organic C stocks were quantified at equivalent soil masses rather than at fixed depths to avoid overestimating SOC stocks in treatments with greater bulk densities (Ellert and Bettany, 1995; Wendt and Hauser, 2013). First, SOC stocks were calculated as the product of the SOC concentration and the mass for each depth increment, scaled to units of mass C per area using the core diameter. We plotted cumulative SOC content against cumulative soil mass for each system at each location and fitted cubic spline functions to the relationships. The cubic spline functions were used to estimate the cumulative SOC content at cumulative soil masses corresponding to those obtained for the 0–15, 15–30, 30–60, and 60 − 90 cm depth increments collected in the two-year system at each location (Wendt and Hauser, 2013). Thus, for each location, the SOC stock of the four-year system was scaled to the same mass of soil sampled in the two-year system. Because the four-year system usually had slightly lower bulk densities than the two-year system (Table 2), adjusting SOC stocks to the equivalent mass of soil sampled in the two-year system increased the size of SOC stocks for the four-year system relative to the fixed depth approach. However, differences in profile SOC stocks between the equivalent mass and fixed depth approaches were minor (< 2 % difference).

2.6. Soil biochemical analyses

We characterized the biochemical composition of soil organic matter by measuring concentrations of carbohydrates and phenols. Carbohydrates were extracted from air-dried and finely ground soil samples in duplicate using a two-step digestion (Martens and Loeffelmann, 2002). First, hemicellulose sugars (arabinose, galactose, glucose, and xylose) were extracted by treating 100 mg of each soil sample with 800 μl 6 M H_2SO_4 for 30 min in test tubes (16 × 100 mm) and diluted with 4.0 ml deionized (DI) water before autoclave digestion for 30 min at 121^0 C. The samples were then washed with two aliquots of 1 ml DI water, centrifuged, and the three supernatants combined and adjusted to pH 5.5–6.5 using NaOH. The extracted and washed samples were dried overnight at 60^{0}C, treated with 300 μl 18 M H_2SO_4 for 30 min, diluted with 3.3 ml DI water, and autoclaved for 30 min at 121^{0}C to release strong acid-extractable glucose, often considered to represent cellulose. The samples were washed with two aliquots of 1 ml DI water, and the supernatants combined and adjusted to pH 5.5–6.5 using NaOH. The extracts were filtered using a 0.2 μm syringe filter and stored frozen prior to analysis. The extracts were analyzed using high-performance anion exchange chromatography with pulsed amperometric detection (Olk, 2008). We used the ratio of galactose plus mannose to arabinose plus xylose (Gal + Man/Arab + Xyl) to represent the relative abundance of microbial to plant carbohydrates (Oades, 1984).

We measured concentrations of phenolic compounds released upon cupric oxide oxidation to quantify the lignin residue content of each soil sample following Hedges and Mann (1979) as modified by Filley et al. (2008). Depending on the sample's SOC concentration, 200 − 500 mg of sample was placed in pressure bombs together with NaOH and CuO. The bombs were purged with Ar gas, sealed, and heated for 3 h at 150° C. The phenols were extracted by repeated ether washes and centrifugations, then filtered and derivatized using bis(trimethylsilyl)trifluoroacetamide. Ethyl vanillin was added to each sample as an internal standard prior to extraction. Gas chromatography flame ionization detection was used to assess concentrations of cinnamic acids (ferulic acid and p-hydroxycoumaric acid), as well as aldehyde, ketone and acid forms of syringyl and vanillyl monomers. We took the sum of these compounds' masses divided by the whole SOC concentration to represent the relative amount of lignin in soils (lignin-VSC/OC) (Kogel-Knabner, 2000). We also used the acid/aldehyde mass ratios of vanillyl and syringyl units to determine the degree of microbial alteration of lignin residues (Ad/Al_v and Ad/Al_s, respectively) (Hedges et al., 1988).

2.7. Soil physical fractionation

We isolated five physical soil organic matter fractions representing different degrees of protection: coarse POM outside microaggregates (cPOM), fine POM outside microaggregates (fPOM), microaggregate-occluded POM (oPOM), easily dispersed silt plus clay (dSilt + Clay), and microaggregate-derived silt plus clay (μSilt + Clay). The POM fractions (i.e., cPOM, fPOM, and oPOM) are considered relatively labile and derived primarily from plant fragments, whereas the MAOM fractions (i.e., dSilt + Clay and μSilt + Clay) are considered relatively stable and derived from both plant and microbial products (Lavallee et al., 2019; Six et al., 2001). Due to physical protection, microaggregate-occluded organic matter (i.e., oPOM and μSilt + Clay) is considered more stable than non-occluded organic matter (i.e., cPOM, fPOM, and dSilt + Clay) (Jastrow et al., 1996). Because the formation of microaggregates occurs as fresh residue decomposes, POM found within microaggregates is usually more microbially-processed than free POM found outside microaggregates (Six et al., 2000).

The first step in the fractionation procedure was to separate three size fractions by partial dispersion using the microaggregate isolator described by Six et al. (2000). Approximately 10 *g* of 8-mm sieved, air-dried soil from each sample were soaked in 50 ml of DI water overnight, then poured onto a 250-μm screen inside a cylinder and reciprocally shaken with 50 metal beads under continuously flowing DI water for eight minutes. Coarse POM outside microaggregates and sand were collected on the 250-μm sieve, while a 53-μm sieve below the 250-μm sieve isolated microaggregates, sand, and fPOM. Easily dispersed silt plus clay was collected in a container below the 53-μm sieve. Following eight minutes of shaking, macroaggregates remaining on the 250-μm sieve were broken up using a metal spatula and their constituents were rinsed through the stacked sieves using a DI wash bottle. The 53 − 250 μm fraction (microaggregates, sand, and fPOM) was wet-sieved by moving the 53-μm sieve up and down in the basin of silt and clay water. The suspension of silt and clay was centrifuged to facilitate the removal of water. The fractions were oven-dried (105^0 C) and weighed.

The second step was a further fractionation of the 53 − 250 μm fraction isolated in the first step. Fine POM outside microaggregates was separated from the microaggregates and sand by density flotation using 1.85 g cm^{-3} sodium polytungstate (Six et al., 2000). The heavy fraction was then dispersed by shaking in 0.5 % sodium hexametaphosphate with ten glass beads for 18 h and passed through a 53-μm sieve to separate the oPOM from the μSilt + Clay. The fractions isolated in the second step were also dried at 105^0 C and weighed.

The cPOM, fPOM, oPOM, dSilt + Clay, and μSilt + Clay fractions were ground using a mortar and pestle and analyzed for total C concentration using dry combustion elemental analysis (Vario Max CN analyzer, Elementar Americas, Mt. Laurel, NJ) following carbonate removal by HCl fumigation (Harris et al., 2001). The SOC concentration of each fraction was multiplied by the proportion of each fraction to total soil mass and divided by the sum of SOC in all fractions to determine the C content of each fraction per unit of total SOC (e.g., frPOM-C/OC). When presenting SOC content of individual fractions, we combined cPOM and fPOM into a single 'free POM' (frPOM) fraction.

The proportion of each fraction by mass, SOC concentration of each fraction, and mass and SOC recoveries are presented in Supplementary Tables 6-8. Mass recovery (i.e., sum of all fraction masses divided by whole sample mass) ranged from 90 to 106 % among the cropping systems, depth increments, and locations. Carbon recovery (i.e., sum of all fraction SOC divided by whole sample SOC) ranged from 97 to 115 % for the top three depth increments, but reached 120-25 % in the deepest layers at Kanawha and Marsden. The high SOC recovery in these deep layers may be due to lower analytical accuracy with very low SOC concentrations associated with the 60 − 90 cm depth increment.

2.8. Statistical analyses

Statistical analysis was performed using linear mixed-effects models in the R package nlme (Pinheiro et al., 2014; R Core Team, 2018). Each location was analyzed separately. When analyzing the soil physical properties (i.e., sand content, clay content, and bulk density), the fixed effects included cropping system, depth increment, and the interaction

Table 3
Estimated average annual C inputs (2003–2014) for two-year and four-year cropping systems at three long- experiments in Iowa, USA. Standard errors are shown in parentheses. Different lowercase letters indicate significantly different average annual C inputs between two crop rotations at a given location ($P < 0.10$).

System	Aboveground input	Belowground input			Manure input	Total input	Proportion of C delivered belowground
		Total belowground	0 − 30 cm	30 − 90 cm			
	(Mg C ha^{-1} yr^{-1})						
				Kanawha			
Two-year	3.19 (0.10) a	1.81 (0.06) b	1.24 (0.04) b	0.32 (0.01) b	–	5.00 (0.17) b	0.36
Four-year	2.73 (0.09) b	2.44 (0.07) a	1.64 (0.05) a	0.48 (0.01) a	–	5.17 (0.16) a	0.47
				Nashua			
Two-year	3.48 (0.16) a	1.97 (0.09) b	1.35 (0.06) b	0.34 (0.02) b	–	5.45 (0.25) a	0.36
Four-year	2.85 (0.12) b	2.62 (0.10) a	1.75 (0.06) a	0.52 (0.02) a	–	5.46 (0.21) a	0.48
				Marsden			
Two-year	3.24 (0.10) a	1.84 (0.05) b	1.27 (0.04) b	0.33 (0.01) b	–	5.08 (0.15) a	0.36
Four-year	2.09 (0.05) b	2.18 (0.07) a	1.45 (0.05) a	0.41 (0.01) a	0.42 (0.04)	4.70 (0.12) b	0.46

of cropping system by depth increment. Depth increment was treated as a categorical variable when analyzing the soil physical properties because these properties did not consistently respond to depth in a linear or parabolic form. Plot nested within block was included as a random effect. When analyzing average aboveground and belowground C inputs, cropping system was included as a fixed effect and year was included as a random effect. Years served as replicates for the analysis of C inputs because we used yield data averaged across field replicates when calculating the C inputs for each treatment. Analysis of variance was used to evaluate the significance of fixed effects. Interactive and main effects that were not significant were removed from models in a stepwise fashion beginning with the cropping system by depth interaction.

Analysis of SOC content was performed separately for each depth increment (0–15, 15–30, 30–60, 60–90, and 0 − 90 cm) to avoid obscuring cropping system effects in specific layers by the high variability of SOC stocks in the entire profile (Kravchenko and Robertson, 2011). Cropping system was included as a fixed effect and block was included as a random effect in the linear mixed-effects models (Pinheiro et al., 2014; R Core Team, 2018). In addition, to account for the effect of textural differences between cropping system treatments on SOC content, we constructed mixed-effects models that included sand and clay contents as fixed effects along with cropping system.

We performed a power analysis for a paired *t*-test using the R pwr package (Champely, 2018) to evaluate the likelihood of statistically detecting differences in whole-profile SOC stocks between cropping systems. We entered the number of replicates, effect size (calculated as the difference in treatment means divided by the pooled standard deviation), and significance level (0.10) and then solved for the power level. We performed a second power analysis to determine the sample size necessary to detect an ecologically-meaningful cropping system effect with 80 % power at a significance level of 0.10. We entered an ecologically-meaningful effect size (calculated using a 20 % difference in SOC stocks and the observed pooled standard deviation), significance level (0.10), and desired power level (80 %), and then solved for the number of experimental replicates.

When analyzing response variables related to organic matter composition and physical fractions (i.e., C/N ratio, Gal + Man/Arab + Xyl, lignin-VSC/OC, Ad/Al_v, Ad/Al_s, μSilt + Clay-C/OC, dSilt + Clay-C/OC, frPOM-C/OC, and oPOM-C/OC), depth was treated as a continuous rather than categorical variable because these variables responded to depth in a linear or parabolic form. Fixed effects included cropping system, depth (i.e., the midpoint of the depth increment), and the interaction of cropping system by depth. Plot nested within block was included as a random effect in all models. Analysis of covariance was used to evaluate the significance of fixed effects. Interactions and main effects that were not significant were removed from models in a stepwise fashion beginning with the highest-order interaction.

Principal component analysis was performed using the R function prcomp (R Core Team, 2018) to assess relationships among soil variables. We conducted principal component analysis for each location using measured soil properties. Biplots were constructed to display the variable loadings and scores for the first two principal components using the fviz function in factoextra (Kassambara and Mundt, 2017). On the biplots, the correlation between two variables is represented by the angle between them, with an acute angle implying positive correlation and an obtuse angle implying negative correlation.

For all linear mixed-effects models, normal distribution of residuals and homogeneity of variances were verified by examining normal probability plots and residuals vs. fitted values. Pairwise comparisons of means were performed using a Tukey test in the lsmeans package (Lenth, 2016), while the glht function in R package multcomp (Hothorn et al., 2008) was used to statistically compare regression coefficients. In an effort to balance Type I vs. Type II errors in the statistical analysis of these field experiments with low replication, we chose to determine significance at the $p = 0.10$ level. All plots, other than the biplots, were created using ggplot2 (Wickham, 2009) using observed means and standard errors.

3. Results

3.1. Average annual C inputs and soil organic C content

Cropping system had little effect on estimated annual total C inputs at the three long-term cropping systems experiments, but had a significant effect on the proportion of C inputs allocated belowground (Table 3). Annual total C inputs were 3 % greater in the four-year than the two-year system at Kanawha, and 8 % greater in the two-year than the four-year system at Marsden ($P < 0.10$). Annual total C inputs were practically identical between cropping systems at Nashua. Although total C inputs were similar between systems, the four-year system delivered 20–35 % more C belowground than the two-year system ($P < 0.10$). The proportion of total C allocated belowground averaged 0.47 in the four-year system and 0.36 in the two-year system (Table 3).

The whole-profile SOC stock was 16 % greater in the four-year system than in the two-year system at Kanawha ($P < 0.10$; Table 4). The percentage increase in SOC in the four-year system relative to the two-year system was relatively consistent among soil layers (14–22 % increase among depth increments) and the effect of cropping system on SOC content was significant in the 0–15 and 60 − 90 cm layers ($P < 0.10$; Table 4). The significance of this effect was maintained with the inclusion of sand and clay content as covariates in the statistical model, meaning that the cropping system effect was significant even after accounting for slight textural differences between the treatments. At Nashua and Marsden, there was no statistically-significant effect of cropping system on SOC content for individual layers (P values ranged from 0.11 to 0.78 at Nashua and from 0.20 to 0.76 at Marsden) or for the entire profile ($P = 0.45$ at Nashua and $P = 0.58$ at Kanawha). Total

Table 4
Soil organic C content by cropping system and depth at three long-term experiments in Iowa, USA. Standard errors are shown in parentheses. Different letters indicate significant differences in soil organic C between cropping systems for a specific location and depth increment ($P < 0.10$).

Depth (cm)	Soil organic C (Mg C ha^{-1})	
	Two-year	Four-year
	Kanawha	
0–15	45.4 (2.31) b	52.7 (0.31) a
15–30	40.3 (4.01) a	47.1 (0.37) a
30–60	38.0 (3.15) a	43.3 (2.54) a
60–90	16.9 (1.57) b	20.7 (2.40) a
Total	141 (9.63) b	164 (7.93) a
	Nashua	
0–15	31.9 (0.96) a	36.3 (1.28) a
15–30	27.6 (3.12) a	29.0 (3.37) a
30–60	22.6 (2.29) a	25.2 (2.72) a
60–90	12.6 (1.06) a	13.7 (0.91) a
Total	94.7 (7.43) a	104 (7.03) a
	Marsden	
0–15	47.4 (4.32) a	43.8 (5.48) a
15–30	42.7 (6.16) a	40.6 (3.06) a
30–60	46.4 (3.36) a	40.4 (3.22) a
60–90	19.5 (2.79) a	21.1 (2.94) a
Total	156 (15.5) a	146 (10.6) a

SOC stocks to 90 cm ranged from 95 to 164 Mg C ha^{-1} among cropping systems and locations. Averaged across systems and locations, 68 % of the total SOC stock to 90 cm was found below 15 cm and 39 % was found below 30 cm.

3.2. Soil organic matter biochemical composition

Soil C/N ratio responded differently to depth and cropping system depending on location. At Kanawha, the C/N ratio exhibited a quadratic response, with values stable and then decreasing with depth for the two-year system ($P < 0.10$ for quadratic coefficient) or increasing and then decreasing with depth for the four-year system ($P < 0.10$ for linear and quadratic coefficients; Fig. 1a and Supplementary Table 9). The linear coefficient was significantly greater for the four-year system than two-year system ($P < 0.10$; Supplementary Table 9), indicating that the C/N ratio increased more with depth in the surface layers of the four-year system than the two-year system. At Nashua, the C/N ratio decreased linearly with depth for both systems ($P < 0.10$ for the linear coefficient; Fig. 1b and Supplementary Table 9). However, the linear coefficient was significantly more negative for the two-year system ($P < 0.10$; Supplementary Table 9), meaning that the C/N ratio decreased more markedly with depth in the two-year system than in the four-year system (Fig. 1b and Supplementary Table 9). At Marsden, the C/N ratio was relatively constant with depth and similar between cropping systems (Fig. 1c and Supplementary Table 9). Averaged across cropping systems and depths, the C/N ratios were greater at Kanawha and Marsden than at Nashua (10.7 at Kanawha and Marsden vs 8.8 at Nashua).

The ratio of microbial- to plant-derived carbohydrates (Gal + Man/Arab + Xyl) increased linearly with depth in all three experiments ($P < 0.10$ for linear coefficients), but there was no significant effect of cropping system on Gal+Man/Arab+Xyl or its response to depth at any location ($P > 0.10$ for cropping system effects on intercepts and linear coefficients; Fig. 1d–f and Supplementary Table 9). The ratios ranged from 0.7 to 1.3, falling between characteristic values for plant polysaccharides (< 0.5) and microbial polysaccharides (> 2.0) (Oades, 1984).

Subsoils were less enriched in lignin than topsoils, with pronounced decreases in lignin-VSC/OC between the surface and approximately 30 cm and slight increases below approximately 60 cm ($P < 0.10$ for linear and quadratic coefficients; Fig. 1g–i). At Kanawha, lignin-VSC/OC was significantly greater near the soil surface and showed a more pronounced decline with depth in the four-year system than the two-year system ($P < 0.10$ for cropping system effects on intercepts and linear coefficients; Fig. 1g and Supplementary Table 9). At Nashua and Marsden, lignin-VSC/OC and its response to depth were similar between the cropping systems ($P > 0.10$ for cropping system effects on intercepts, linear, and quadratic coefficients; Supplementary Table 9). Lignin-VSC/OC ranged from 0.2 to 1.8 g VSC 100 g SOC^{-1} among cropping systems, depths, and locations.

The effects of cropping system and depth on Ad/Al_v, which is positively associated with lignin oxidation, differed depending on location (Fig. 2a–c). There were no main effects or interactive effects of cropping system and depth on Ad/Al_v at Kanawha (Fig. 2a). A positive linear trend was observed for both systems at Nashua ($P < 0.10$ for linear coefficients), and the response was more pronounced for the two-year system than the four-year system ($P < 0.10$ for cropping system effect on linear coefficient; Fig. 2b and Supplementary Table 10). The two-year system at Marsden showed a significant quadratic response, where Ad/Al_v was greatest at intermediate depths and lowest in the shallowest and deepest layers ($P < 0.10$ for linear and quadratic coefficients; Fig. 2c and Supplementary Table 10), whereas the four-year system at Marsden showed a positive linear response to depth (Fig. 2c). Average Ad/Al_v values among cropping systems, depths, and locations ranged from 0.35 to 1.1. There were no main effects or interactive effects of cropping system and depth on Ad/Al_s, which ranged from 0.38 to 0.95 ($P > 0.10$; Fig. 2d–f and Supplementary Table 10).

3.3. Distribution of soil organic C in physical fractions

The majority (50–75 %) of SOC was found in the µSilt + Clay at all three locations (Fig. 3a–c). Most of the remaining SOC (25–45 %) was recovered from the dSilt + Clay, so that the combined MAOM fractions made up 94–99 % of total SOC. The percentage of SOC recovered as µSilt + Clay-C was greatest in the topsoil and decreased with depth in a linear or quadratic pattern ($P < 0.10$ for linear coefficients at Kanawha and Nashua and $P < 0.10$ for quadratic coefficients at Nashua and Marsden; Fig. 3a–c and Supplementary Table 11). The percentage of SOC as dSilt + Clay-C increased with depth ($P < 0.10$ for linear coefficients at Kanawha and Marsden and $P < 0.10$ for quadratic coefficient at Nashua; Fig. 3d–f and Supplementary Table 11). The percentages of SOC within these MAOM fractions and their response to depth were similar between cropping systems ($P > 0.10$ for cropping system effects on intercepts, linear and quadratic coefficients; Supplementary Table 11).

Particulate organic matter outside microaggregates (frPOM-C) made up between 0.3 and 5 % of the total SOC across cropping systems, depths, and locations and exhibited a quadratic response to depth at Nashua and Marsden ($P < 0.10$ for linear and quadratic coefficients; Fig. 4a–c and Supplementary Table 11). The percentage of SOC as frPOM-C generally decreased with depth in the surface layers and increased with depth below approximately 60 cm (Fig. 4a–c). Microaggregate-occluded POM-C made up between 0.01 and 0.06 % of total SOC across cropping systems, depths, and locations (Fig. 4d–f). At Kanawha, oPOM-C/OC exhibited a quadratic response, with values stable and then increasing with depth for the two-year system ($P < 0.10$ for quadratic coefficient) or decreasing and then increasing with depth for the four-year system ($P < 0.10$ for linear and quadratic coefficients; Fig. 4d and Supplementary Table 11). At Nashua and Marsden, similar quadratic responses of oPOM-C/OC with depth were observed, but no cropping system effects were detected ($P > 0.10$ for cropping system effects on intercepts, linear, and quadratic coefficients; Supplementary Table 11).

3.4. Multivariate analysis of soil properties

The first three principal components derived from the principal

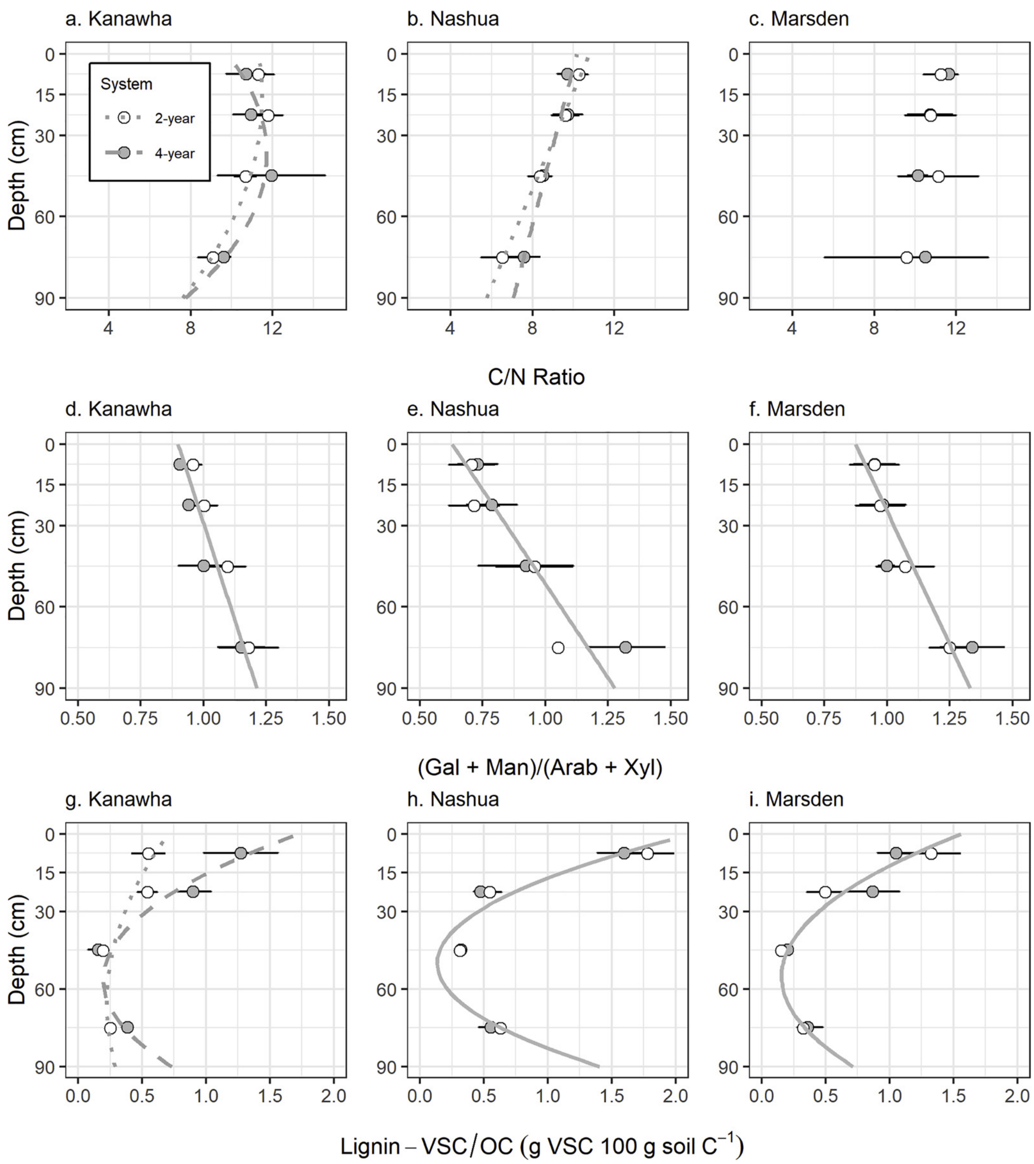

Fig. 1. The C/N ratio, galactose plus mannose to xylose plus arabinose ratio, and C-normalized lignin residues (lignin-VSC/OC) by cropping system and depth at three long-term experiments in Iowa, USA. Points are plotted at the mid-depth of each sampled layer. Error bars are ± one SE. Curves are linear or quadratic regressions fitted to the data (shown only where there was a significant quadratic or linear depth effect).

component analysis explained 72–81 % of variance in the data sets (Supplementary Table 12). For Kanawha and Nashua, the variables that contributed the most to the first principal component included sand percentage, bulk density, Gal + Man/Arab + Xyl, and oPOM-C/OC in the positive direction, and SOC concentration, silt percentage, and C/N ratio in the negative direction (Fig. 5a–b and Supplementary Table 12). Clay percentage also contributed to the first principal component for Kanawha in the negative direction (Fig. 5a and Supplementary Table 12). For Marsden, the variables that contributed the most to the first principal component were SOC concentration and μSilt + Clay-C/OC in the positive direction, and bulk density, dSilt + Clay-C/OC, frPOM-C/OC, and oPOM-C/OC in the negative direction (Fig. 5c and Supplementary Table 12). Soil organic C concentration, lignin-VSC/OC, and the physical fractions contributed to the second principal component for Kanawha and Nashua, while particle size classes, Gal + Man/Arab + Xyl, and C/N ratio were more important contributors to the second principal component at Marsden (Fig. 5a–c and Supplementary Table 12). The Ad/Al ratios of vanilyl and syringyl contributed mostly to the third principal component for all three locations (Supplementary Table 12). In general, the shallow depth increments were positively associated with SOC concentration, lignin-VSC/OC, C/N ratio, and μSilt + Clay-C/OC while deep depth increments were positively associated with bulk density, sand percentage, Gal + Man/Arab + Xyl, dSilt + Clay-C/OC, and oPOM-C/OC (Fig. 5a–c).

4. Discussion

4.1. Cropping system effects

The purpose of this study was to determine whether four-year, alfalfa-inclusive cropping systems deliver more C belowground than two-year maize-soybean systems, and, if so, whether the greater belowground C inputs result in deep SOC storage. We found that average annual belowground C inputs were 20–35 % greater in the four-year systems despite total C inputs within 10 % of the corresponding two-year systems (Table 3). Although considerable uncertainty exists in estimation of belowground C inputs (Bolinder et al., 2007), our sensitivity analysis showed that the four-year system had greater belowground C inputs than the two-year system across a range of different shoot/root ratios representing ± one standard deviation of reported

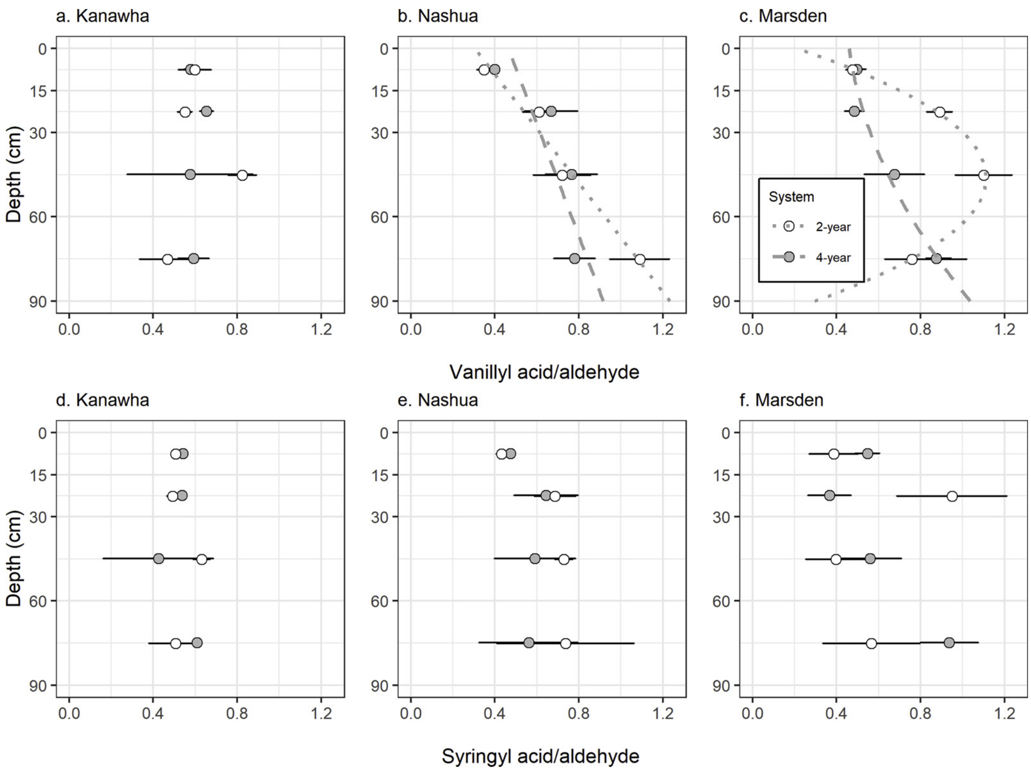

Fig. 2. The acid/aldehyde ratios of vanillyl and syringyl structural units by cropping system and depth at three long-term experiments in Iowa, USA. Points are plotted at the mid-depth of each sampled layer. Error bars are ± one SE. Curves are linear or quadratic regressions fitted to the data (shown only where there was a significant depth effect).

values (Supplementary Table 5). The only case of the four-year system having lower belowground C inputs than the two-year system in our sensitivity analysis was when annual crops had lower shoot/root ratios and alfalfa had higher shoot/root ratios than estimated by Bolinder et al. (2007).

Soil organic C content was 16 % greater in the four-year system than in the two-year system at Kanawha (Table 4). Assuming that the two- and four-year systems began with the same SOC stocks, the four-year system gained SOC at a rate of 0.38 Mg C ha^{-1} yr^{-1} to 90 cm relative to the two-year system averaged over the experiment duration. For the top 30 cm, the relative accumulation rate was 0.24 Mg C ha^{-1} yr^{-1}, which is similar to that reported for diversified crop rotations in a global data synthesis of surface SOC changes (mean = 0.20 Mg C ha^{-1} yr^{-1}) (West and Post, 2002). However, the greater SOC stock in the four-year than two-year system measured at a single time point does not necessarily indicate a net increase in SOC stock over time in the four-year system.

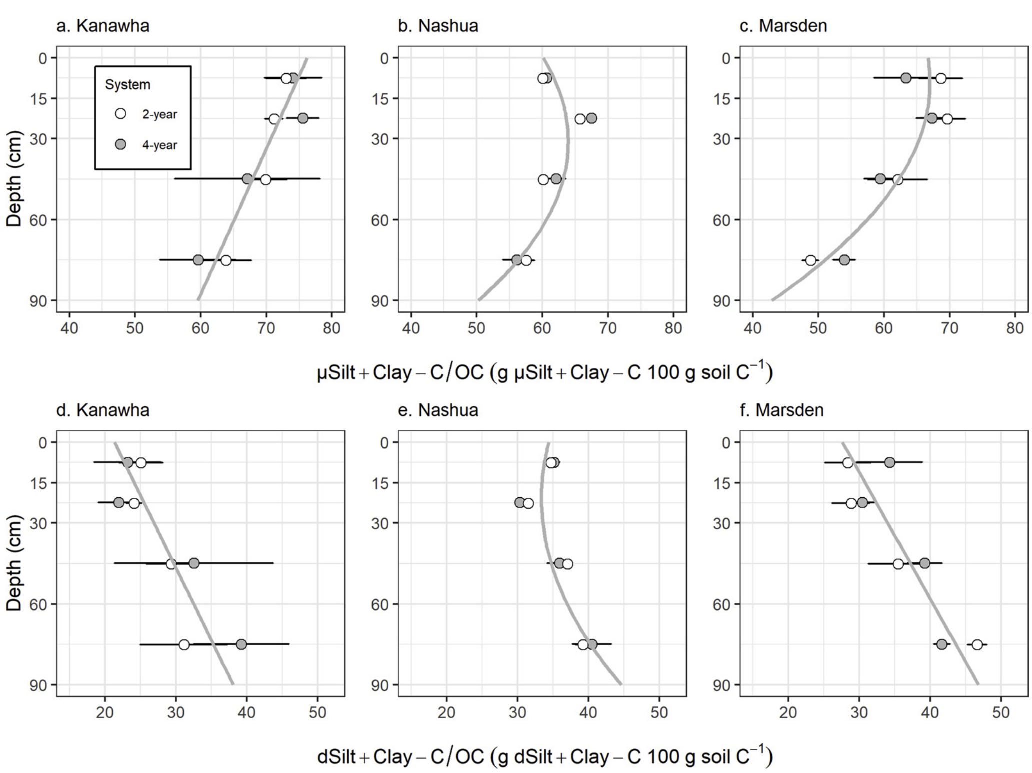

Fig. 3. Percentage of total soil organic C in microaggregate-derived silt plus clay (μSilt + Clay) and easily-dispersed silt plus clay (dSilt + Clay) by depth and cropping system at three long-term experiments in Iowa, USA. Points are plotted at the mid-depth of each sampled layer. Error bars are ± one SE. Curves are linear or quadratic regressions fitted to the data (shown only where there was a significant quadratic or linear depth effect).

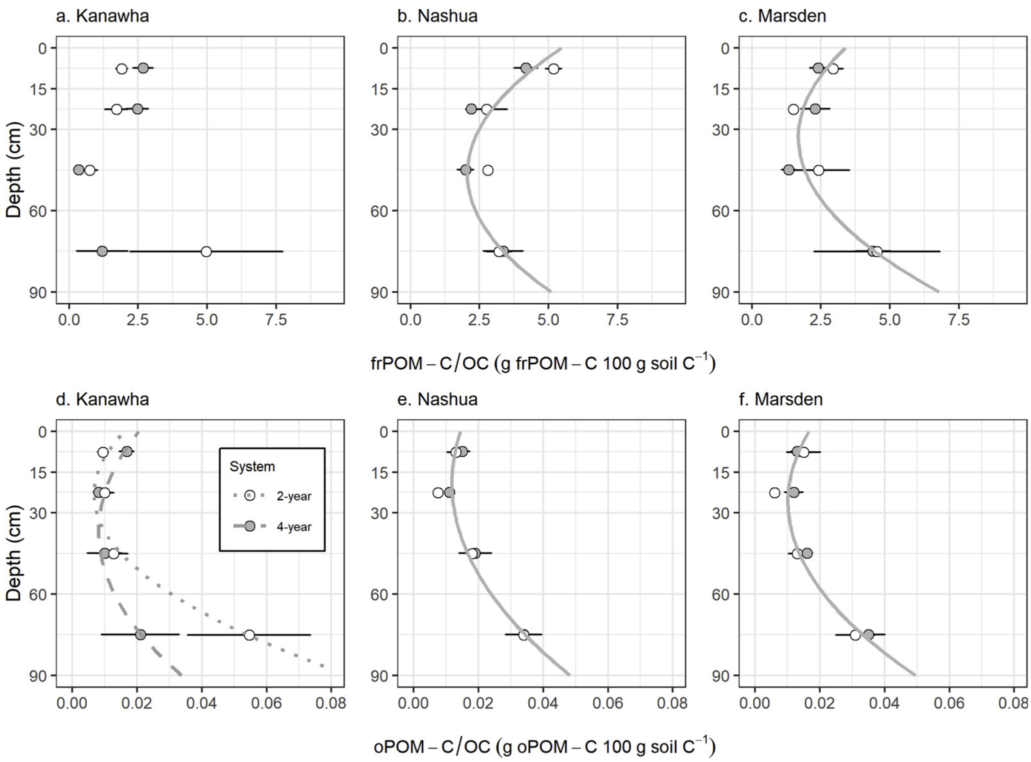

Fig. 4. Percentage of total soil organic C in free particulate organic matter (frPOM) and microaggregate-occluded particulate organic matter (oPOM) by depth and cropping system at three long-term experiments in Iowa, USA. Points are plotted at the mid-depth of each sampled layer. Error bars are ± one SE. Curves are linear or quadratic regressions fitted to the data (shown only where there was a significant quadratic or linear depth effect).

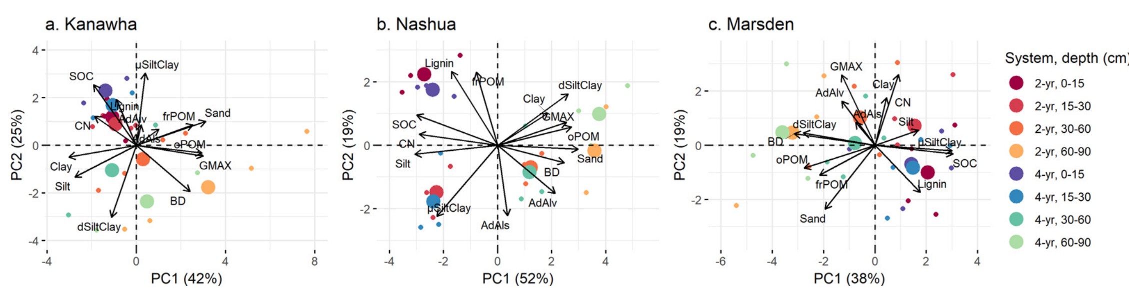

Fig. 5. Biplots of the first and second principal components based on soil data collected at three long-term cropping systems experiments in Iowa, USA. SOC = soil organic C concentration; Sand, Silt, and Clay = percentages of each mineral size class; BD = bulk density; CN = carbon to nitrogen ratio; GMAX = galactose plus mannose to xylose plus arabinose ratio; Lignin = carbon-normalized lignin residues; AdAlv and AdAls = acid/aldehyde mass ratios of vanillyl and syringyl units; μSiltClay, dSiltClay, frPOM, and oPOM = percentages of total soil organic C in microaggregate-derived silt plus clay, easily-dispersed silt plus clay, free particulate organic matter, and microaggregate-occluded particulate organic matter, respectively. Enlarged points represent the mean of each group.

The greater SOC stock measured in the four-year system at Kanawha may simply reflect a reduction or cessation in SOC losses relative to the two-year system rather than a net gain in SOC over time (Sanderman and Baldock, 2010; Sanford et al., 2012).

At Nashua and Marsden, SOC stocks were not significantly different between cropping systems. The lack of a cropping system effect at these locations may be due to the shorter experimental durations of these studies (Table 2). In addition, at Marsden, the four-year system had slightly lower total C inputs (despite manure inputs) and more intensive tillage (i.e., moldboard plowing rather than chisel tillage) than the two-year system, factors that may have offset the benefits of greater belowground inputs in the four-year system at this site.

Although we did not detect a statistically significant effect of cropping system on SOC stocks at Nashua and Kanawha, we do not interpret this to mean that cropping system definitively had no effect. Due to large inherent SOC variation, particularly in subsoils, many replicates are required to detect statistical differences in whole-profile SOC stocks (Kravchenko and Robertson, 2011). For the present study, a post-hoc power analysis revealed that the likelihood of detecting an effect as significant was only 14–21% for the three sites (> 80 % is desirable). We had 80 % power to detect a 36–83 % difference in SOC stock. Based on measured standard deviations in profile SOC stocks, we would have needed 6 and 9 replicates at Nashua and Marsden, respectively, to statistically detect a 20 % difference in SOC due to cropping system ($P < 0.10$; Power = 80 %).

Our results were partially consistent with the hypothesis that soil organic matter would be more enriched in fresh plant-derived C, particularly at depth, in the four-year than the two-year system due to greater root C inputs in the four-year system. In the four-year system at Kanawha, the C/N ratio increased significantly with depth before decreasing in deeper layers, whereas in the two-year system, the C/N ratio was constant near the surface before declining in deeper layers (Fig. 1a). Also, at Nashua, the C/N ratio decreased less markedly with depth in the four-year than two-year system as indicated by a significantly less negative slope (Fig. 1b and Supplementary Table 9). Second, at Nashua and Marsden, lignin oxidation (as measured by Ad/Al_v) increased less with depth in the four-year than two-year system (Fig. 2b and c). The lower degree of lignin oxidation in subsoil horizons of four-year than two-year systems may reflect greater inputs of root-derived lignin or less output through lignin decomposition in these layers. Greater lignin-VSC/OC in surface soil of the four-year than two-year system at Kanawha (Fig. 1g) also indicates greater plant-derived and biochemically-recalcitrant C in topsoil of the four-year system and aligns with findings by Gregorich et al. (2001) that aromatic C was greater under maize-oat-alfalfa-alfalfa rotations than under monoculture maize. Apart from these cases, effects of cropping system on biochemical variables were minimal, suggesting that the greater delivery of root C in the four-year systems was accompanied by a proportional increase in microbial processing throughout the profile. This finding supports recent observations of high C turnover in both topsoils and subsoils when organic substrate is added (Jones et al., 2018).

Although recent research indicates that increasing root-C inputs can stimulate aggregation in deep soil layers (Baumert et al., 2018), the results of our physical fractionation indicate that integrating alfalfa into a cropping system has little impact on SOC stabilization through microaggregation. At Nashua and Marsden, the distribution of SOC among fractions was quite similar between cropping systems at all depths (Figs. 3 and 4). At Kanawha, the response of oPOM-C/OC to depth differed between cropping systems, as indicated by significantly different linear coefficients (Fig. 3d and Supplementary Table 11). This cropping system effect may be explained by a textural difference between the two systems at Kanawha: the deepest sampled layer of the two-year system had 53 % higher sand content than the same layer of the four-year system and would therefore be expected to store more C in the sand-sized POM fractions (Table 2) (Angst et al., 2018).

It is important to recognize that the integration of a deep-rooted perennial forage crop like alfalfa into a crop rotation is usually accompanied by changes in nutrient management and tillage practices as well, which may affect SOC content, composition, and distribution with depth. Because alfalfa is fed to ruminant livestock, alfalfa-inclusive cropping systems often derive a portion of nutrient inputs from manure (Poffenbarger et al., 2017; Sulc and Tracy, 2007). Manure contains C along with N, P, and other nutrients and has been shown to enrich soil organic matter in POM and plant-derived sugars and decrease the soil C/N ratio (Xie et al., 2014). The inclusion of a perennial crop is also usually accompanied by a reduction in tillage frequency (because tillage is not performed during the perennial crop phase) but an increase in tillage intensity (because terminating the perennial crop may require inversion rather than vertical tillage), which can affect decomposition rates and SOC vertical distribution (Angers and Eriksen-Hamel, 2008). The differential use of manure and inversion tillage in two-year and four-year systems occurred in the Marsden experiment, but not in the Kanawha and Nashua experiments (Table 1). With that said, the SOC content, distribution of SOC among fractions, and the changes in organic matter properties by depth and cropping system were not strikingly different between Marsden and the other two locations.

4.2. Vertical patterns

Our results on biochemical properties show that SOC becomes increasingly microbially-processed with greater depth in the soil. We found negative responses of C/N ratio and lignin-VSC/OC and positive responses of Gal + Man/Arab + Xyl and Ad/Al_v with depth, indicating greater enrichment of microbial compounds and greater extent of decomposition in the subsoil than in the topsoil (Figs. 1 and 2). The effect of depth on these variables was most pronounced at Nashua, the location that likely had the fastest C turnover (i.e., the location with the greatest C inputs and lowest SOC stocks). The vertical patterns in biochemical properties are consistent with other reports of decreasing C/N ratio and increasing microbial-derived amino sugars with greater depth (Rumpel and Kögel-Knabner, 2011) and likely reflect the decreasing inputs of shoot- and root-C and the increasing contribution of old, recycled SOC either originally derived from roots or transported to subsoils from surface layers.

The vast majority of SOC (> 90 %) was associated with silt and clay particles at all soil depths, a finding that is consistent with results from other fractionation studies conducted on finely-textured Mollisols of the Midwest U.S. (Brown et al., 2014; Cates and Ruark, 2017; Lazicki et al., 2016). However, the distribution of MAOM between the microaggregate-associated silt plus clay (μSilt + Clay) and easily-dispersed silt plus clay (dSilt + Clay) shifted with depth, probably reflecting decreasing amounts of plant- and microbial-derived C and associated biological activity. At all three sites, μSilt + Clay-C/OC either declined over the whole profile (Kanawha) or declined below ∼30 cm depth (Nashua and Marsden) as dSilt + Clay-C/OC increased (Fig. 3). Microaggregates form within stable macroaggregates as organic fragments inside the macroaggregates degrade, producing agents that bind soil particles (Six et al., 2000). The decline in μSilt + Clay-C/OC with depth corresponds to declining biological activity, (e.g., lower abundance of roots, fungi, and earthworms) and declining concentrations of organic binding agents, which contribute to stable aggregate formation (Barois et al., 1993; Jarvis et al., 1982; Shipitalo and Protz, 1988). The importance of organic binding agents for stable microaggregate formation is underscored by the close association between SOC concentration and μSilt + Clay-C/OC revealed by our principal component analysis (Fig. 5).

The vertical patterns of most of the biochemical properties suggest a decreasing contribution of fresh plant-derived C and increasing contribution of microbially-processed C to SOC with greater depth. However, the quadratic responses of lignin-VSC/OC, frPOM-C/OC, and oPOM-C/OC with depth (Figs. 1 and 4) show that these largely plant-derived fractions of SOC made up a greater proportion of total SOC in the deepest layer than in the upper portion of the subsoil. This apparent contradiction may be explained by the fact that the lignin and POM fractions make up only a small portion of total SOC in these soils and so an increase in their proportional abundance may have only a minor effect on the soil's overall biochemical composition. In other studies, the presence of fresh, plant-derived C in subsoils has been attributed to its physical separation from microbes and/or to unfavorable oxygen, temperature, and moisture conditions for microbes that effectively preserve otherwise labile C at depth (Baumann et al., 2013; Rumpel and Kögel-Knabner, 2011; Salome et al., 2010; Wordell-Dietrich et al., 2017). Our finding that Ad/Al_v decreased (i.e., the lignin became less oxidized) from the upper subsoil to the lower subsoil in the Marsden two-year system lends some support for this explanation. However, it is also important to note that, at all three locations, the greater contribution of lignin-VSC and POM-C to SOC in the deepest layers corresponded to a larger sand fraction (Table 2), which is the mineral fraction associated with POM. When expressed per mass of sand, there was little difference in the amount of lignin-VSC and POM-C between the third and fourth depth increments (0.006 vs. 0.005 mg lignin-VSC 100 g $sand^{-1}$ and 0.0420 vs. 0.0416 g total POM-C 100 g $sand^{-1}$, respectively, averaged across systems and sites). The similarity of sand-normalized lignin-VSC and POM-C between the third and fourth depth increments suggests that the enrichment of these fractions in the deepest layer was not due to slower decomposition of plant inputs but simply due to a larger sand fraction, consistent with findings by Angst et al. (2018).

5. Conclusion

Compared with two-year maize-soybean systems, four-year alfalfa-inclusive systems delivered more C inputs belowground, resulting in greater SOC in topsoils and subsoils over the long-term at one of three locations. However, cropping system diversification had a minimal effect on the biochemical indicators or the allocation of SOC among physical fractions, suggesting that the composition and stability of SOC are similar between the two systems. In both of the systems at all three locations, the soil organic matter became more microbially-processed and the proportion of MAOM found in microaggregates declined with depth. We conclude that adoption of cropping systems with enhanced belowground C inputs may increase total profile SOC, but the effect is minimal and inconsistent; furthermore, it has minor impact on the vertical distribution, biochemical composition, or stability of SOC in

Mollisols of the Midwest U.S.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.agee.2019.106810.

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